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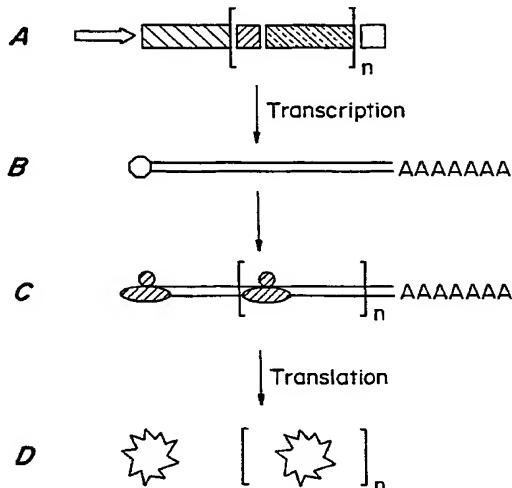
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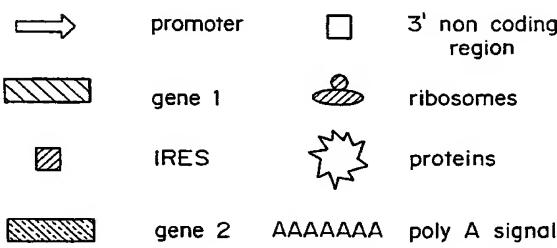
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[Continued on next page]

(54) Title: PLANT MULTI-GENE EXPRESSION CONSTRUCTS



(57) Abstract: Methods and constructs are provided for the introduction of multiple genes into plants using a single transformation event. Coordinated expression of genes in the cassette, producing proteins with native amino acid sequences, is achieved by production of one polycistronic mRNA that contains separate translation initiation signals for each enzyme coding region. Bicistronic constructs contain a single 5' promoter, protein encoding sequence 1, an IRES, protein encoding sequence 2, and a single 3' polyadenylation sequence. For polycistronic constructs, additional cassettes of protein encoding sequences, in which each coding region is preceded by an IRES, can be inserted between protein encoding sequence 2 and the polyadenylation sequence. The methods and constructs are useful for creating plants with stacked input traits (e.g., glyphosate tolerant plants producing BT toxin) and/or value added products (e.g., the production of PHAs in plants).



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## PLANT MULTI-GENE EXPRESSION CONSTRUCTS

### Background of the Invention

The present invention generally relates to constructs for transforming  
5 plants, in particular plants useful in the production of polymers.

Genetic engineering of plant crops to produce stacked input traits,  
such as tolerance to herbicides and insect resistance, or value added products,  
such as polyhydroxyalkanoates (PHAs), requires the expression of multiple  
foreign genes. The traditional breeding methodology used to assemble more  
10 than one gene within a plant requires repeated cycles of producing and  
crossing homozygous lines, a process that contributes significantly to the  
cost and time for generating transgenic plants suitable for field production  
(Hitz, *Current Opinion in Plant Biology*, 2:135-38 (1999). This cost could  
be drastically reduced by the insertion of multiple genes into a plant in a  
15 single transformation event.

The creation of a single vector containing cassettes of multiple genes,  
each flanked by a promoter and polyadenylation sequence, allows for a  
single transformation event but can lead to gene silencing if any of the  
promoter or polyadenylation sequences are homologous (Matzke, et al., in  
20 *Homologous Recombination and Gene Silencing in Plants* (Paszkowski, ed.)  
pp. 271-300 (Kluwer Academic Publishers, Netherlands, 1994)). Multiple  
unique promoters can be employed, but coordinating the expression is  
difficult. Researchers have coordinated the expression of multiple genes  
from one promoter by engineering ribozyme cleavage sites into multi-gene  
25 constructs such that a polycistronic RNA is produced that can subsequently  
be cleaved into a monocistronic RNA (see U.S. Patent No. 5,519,164).  
Multiple genes have also been expressed as a polyprotein in which coding  
regions are joined by protease recognition sites (Dasgupta, et al., *The Plant  
Journal*, 16:107-16 (1998)). A co-expressed protease releases the individual  
30 enzymes but often leaves remnants of the protease cleavage site that may  
affect the activity of the enzymes.

The majority of eukaryotic mRNAs are thought to be translated by a scanning ribosome mechanism in which ribosomes can only gain access to translation initiation sites via the 5'-end of the mRNA (Bailey-Serres, *Trends in Plant Science*, 4:142-47 (1999)). A cap structure at the extreme 5' end of 5 the mRNA plays an important role in the initial binding of the ribosome. Several RNAs of viral origin do not contain a 5' cap structure yet can be translated efficiently by the eukaryotic host (*Id.*). This mechanism of cap-independent initiation of protein synthesis is referred to as internal initiation and the sequence promoting the initiation is called an internal ribosome entry 10 site (IRES) (Belsham & Sonenberg, *Microbiol. Rev.*, 60:499-511 (1996)). The internal initiation of protein synthesis is independent of the location of the IRES sequence within the mRNA and does not require the presence of any viral proteins (*Id.*). The sequences used by these viruses therefore can be manipulated to create artificial expression cassettes capable of cap- 15 independent translation of multiple gene coding regions in plants.

The use of IRES sequences in plants for the expression of multiple genes to enhance input traits, or for multi-gene expression for the formation of natural or novel plant products has not been demonstrated. *In vitro* studies with bicistronic constructs containing the 5'-UTR of the crucifer-infecting 20 Tobamovirus have resulted in the successful translation of both coding sequences (Ivanov, et al., *Virology*, 232:32-43 (1997)). Constructs containing several viral sequences for the enhanced expression of a single foreign gene in plants also have been disclosed in PCT WO 98/55636, wherein an IRES signal is used to promote expression of a marker gene for 25 selection of the transgenic plant.

The expression of multiple enzymes is useful for altering the metabolism of plants to increase, for example, the levels of nutritional amino acids (Falco, et al., *Bio/Technology* 13:577 (1995)), to modify lignin metabolism, to modify oil compositions (Murphy, *TIBTECH* 14:206-13 30 (1996)), to modify starch biosynthesis, or to produce polyhydroxyalkanoate polymers (Huisman & Madison, *Microbiol. & Mol. Biol. Rev.* 63:21-53 (1999)). In order to produce PHAs, it is desirable to divert carbon from

normal plant metabolism by the expression of two or more recombinant proteins. PHAs of different compositions and properties can be produced in plants depending on the substrate specificity of the engineered enzymes, the tissues in which these enzymes are expressed, and the pathways from which carbon is diverted (Huisman & Madison, *Microbiol. & Mol. Biol. Rev.* 5 63:21-53 (1999)).

Prior to producing PHAs from plants on an industrial scale, polymer production in crops of agronomic value must be optimized. Preliminary studies in some crops of agronomic value have been performed including 10 PHB production in maize cell suspension cultures and in the peroxisomes of intact tobacco plants (Hahn, J., Ph.D. Thesis, University of Minnesota (Feb. 1998)), as well as PHB production in transgenic canola and soybean seeds (PCT WO 98/00557). In these studies, however, the levels of polymer observed were too low for economical production of the polymer.

15 It is therefore an object of the present invention to provide DNA constructs for the improved insertion of multiple genes into a plant in a single transformation event.

It is another object of this invention to provide enhanced, cost-effective methods for developing transgenic plants for the production of 20 polymers, particularly polyhydroxyalkanoates.

#### **Summary of the Invention**

Methods and constructs are provided for the introduction of multiple genes into plants using a single transformation event. In particular, the construction of multi-gene expression cassettes containing a single promoter 25 and a single polyadenylation signal is provided. Coordinated expression of genes in the cassette, producing proteins with native amino acid sequences, is achieved by production of one polycistronic mRNA that contains separate translation initiation signals for each enzyme coding region. The described arrangement of genes allows the insertion of multiple genes into a plant 30 using a single transformation event. This methodology for the insertion and expression of multiple genes encoding metabolic pathways is useful for

producing value added products, as well as for engineering plants to express multiple input traits.

Creation of polycistronic internal ribosome entry site (IRES) containing vectors is useful for reducing the complexity of the traditional breeding methodology required to make the transgenic plant agronomically useful. Bicistronic constructs contain a single 5' promoter, gene 1, an internal ribosome entry site (IRES), gene 2, and a single 3' polyadenylation sequence. For polycistronic constructs, additional cassettes of genes, in which each coding region is preceded by an IRES, can be inserted between gene 2 and the polyadenylation sequence. The methods and constructs are useful for creating plants with stacked input traits (e.g., glyphosate tolerant plants producing BT toxin) and/or value added products (e.g., the production of PHAs in plants).

#### Brief Description of the Drawings

Figures 1a-d are illustrations of multi-gene expression using internal ribosome entry sites, where Fig. 1a shows a cassette for multi-gene expression; bicistronic construct (n=1), polycistronic construct, (n>1); Fig. 1b shows bi- or poly-cistronic mRNA with one 5' cap and one polyadenylation signal; Fig. 1c shows binding of ribosomes to bicistronic (n=1) or polycistronic (n>1) mRNA constructs, where ribosomes initiate translation at 5' capped portion of mRNA and at all internal ribosome initiation sites downstream of the 5' cap; and Fig. 1d shows translation of bi- or polycistronic mRNA to produce proteins.

Figure 2 is an illustration of short and medium chain length PHA production from fatty acid  $\beta$ -oxidation pathways.

Figure 3 is an illustration of bicistronic construct for testing *in vivo* cap independent translation in *Arabidopsis* protoplasts.

Figure 4 is an illustration of medium chain length PHA production from fatty acid biosynthesis pathways.

Figure 5 is an illustration of a construct for medium chain length PHA production in chloroplasts from fatty acids containing two promoters and two polyadenylation sequences.

Figures 6a-c are illustrations of IRES Constructs for medium chain length PHA production, wherein Fig. 6a shows a construct for medium chain length PHA production in chloroplasts from fatty acid biosynthesis; Fig. 6b shows a construct for medium chain length PHA production in the peroxisomes of leaves using fatty acid  $\beta$ -oxidation; and Fig. 6c shows a construct for medium chain length PHA production in the cytosol of oil seeds using fatty acid  $\beta$ -oxidation.

Figures 7a-f are illustrations of constructs for short chain PHA production from acetyl CoA in plant tissues, wherein Fig. 7a shows a construct expressing reductase and synthase in cytosol; Fig. 7b shows a construct expressing reductase, synthase, and thiolase in cytosol; Fig. 7c shows a construct targeting reductase and synthase to chloroplasts or plastids; Fig. 7d shows a construct targeting reductase, synthase, and thiolase to chloroplasts or plastids; Fig. 7e shows a construct for targeting reductase and synthase to peroxisomes; and Fig. 7f shows a construct for targeting reductase, synthase, and thiolase to peroxisomes.

Figures 8a-c are illustrations of constructs for cytosolic short chain PHA production in seeds from fatty acids using cytosolic fatty acid  $\beta$ -oxidation, wherein Fig. 8a shows a construct for expressing acyl CoA oxidase, a synthase with specificity for short chain substrates, an  $\alpha$ -subunit of  $\beta$ -oxidation, a  $\beta$ -subunit of  $\beta$ -oxidation, and a reductase; Fig. 8b shows a construct for expressing an acyl CoA oxidase, a synthase accepting short chain substrates, an  $\alpha$ -subunit of  $\beta$ -oxidation, a  $\beta$ -subunit of  $\beta$ -oxidation, a reductase, and a catalase; and Fig. 8c shows a construct for expressing an acyl CoA oxidase, a synthase expressing short chain substrates, an  $\alpha$ -subunit of  $\beta$ -oxidation, a  $\beta$ -subunit of  $\beta$ -oxidation, a reductase, and a thiolase.

Figure 9 is an illustration of a construct for producing a plant that is tolerant to glyphosate and that produces *Bacillus thuringiensis* toxin.

#### Detailed Description of the Invention

Optimization of PHA production in crops of agronomic value requires the screening of multiple enzymes, targeting signals, and sites of production until a high yielding route to the polymer with the desired

composition is obtained. This is a task which can be simplified if multiple genes are inserted in a single transformation event. The usefulness of bi- or poly-cistronic vectors to produce PHAs in plants is described herein.

Expression cassettes are provided in which viral IRES sequences are utilized for multi-gene translation. For example, the strategy for utilizing the expression cassettes for producing multiple proteins from a single polycistronic mRNA is outlined in Figure 1. The expression cassettes contain a single promoter at the 5'-end of the construct (Fig. 1a). The promoter may be inducible, constitutive, or tissue specific. The first gene to be expressed is placed directly behind the promoter. If necessary, this gene may be preceded or followed by a peptide signal that targets the protein to a particular compartment of the cell. A viral IRES sequence that is able to initiate cap-independent translation in plants is placed immediately behind the first gene. The source of the IRES sequence may be from any of the viruses capable of initiating cap-independent translation of mRNA. The IRES sequence is followed by a second gene. For targeting of this gene to a certain organelle, a peptide signal can be fused to the coding sequence of the gene. After the second gene, the IRES-gene sequence can be repeated as often as desired for expression of multiple proteins in the same cell (Fig. 1a, n > 1). For multi-IRES containing constructs, it may be useful to use IRES elements from different sources. After the sequence of the last gene to be expressed, a polyadenylation signal must be inserted. This arrangement of genetic information will allow the formation of one polycistronic mRNA (Fig. 1b). Ribosomes can bind independently at both the 5' end of the mRNA and at the IRES sequences allowing independent translation of all protein coding sequences in the polycistronic mRNA (Figs. 1c and 1d).

## I. Materials

### Plant Transformation Vectors

The DNA constructs provided herein include transformation vectors capable of introducing transgenes into plants. There are many plant transformation vector options available (e.g., Gene Transfer to Plants, Potrykus & Spangenberg, eds., Springer-Verlag Berlin Heidelberg New York

(1995); Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins, Owen & Pen, eds., John Wiley & Sons Ltd. England (1996); and Methods in Plant Molecular Biology-a laboratory course manual, Maliga, et al., eds., Cold Spring Laboratory Press, New York 5 (1995)). In general, plant transformation vectors comprise one or more coding sequences of interest under the transcriptional control of 5' and 3' regulatory sequences, including a promoter, a transcription termination and/or polyadenylation signal and a selectable or screenable marker gene. The usual requirements for 5' regulatory sequences include a promoter, a 10 transcription initiation site, and a RNA processing signal. 3' regulatory sequences include a transcription termination and/or a polyadenylation signal.

#### Plant Promoters

A large number of plant promoters are known and result in either 15 constitutive, or environmentally or developmentally regulated expression of the gene of interest. Plant promoters can be selected to control the expression of the transgene in different plant tissues or organelles for all of which methods are known to those skilled in the art (Gasser & Fraley, *Science* 244:1293-99 (1989)). Examples of suitable constitutive plant 20 promoters include the cauliflower mosaic virus 35S promoter (CaMV) and enhanced CaMV promoters (Odell et al., *Nature*, 313:810 (1985)), actin promoter (McElroy et al., *Plant Cell* 2:163-71 (1990)), AdhI promoter (Fromm et al., *Bio/Technology* 8:833-39 (1990); Kyozuka et al., 1991, *Mol. Gen. Genet.* 228:40-48), ubiquitin promoters, the Figwort mosaic virus 25 promoter, mannopine synthase promoter, nopaline synthase promoter and octopine synthase promoter. Useful regulatable promoter systems include, for example, spinach nitrate-inducible promoter, heat shock promoters, small subunit of ribulose biphosphate carboxylase promoters and chemically inducible promoters (see U.S. Patents No. 5,364,780; No. 5,364,780; No. 30 5,777,200).

*Tissue Specific Promoters*

In some embodiments it may be preferable to express the transgenes only in the developing seeds. Promoters suitable for this purpose include the napin gene promoter (U.S. Patent No. 5,420,034 and No. 5,608,152), the 5 acetyl-CoA carboxylase promoter (U.S. Patent No. 5,420,034 and No. 5,608,152), 2S albumin promoter, seed storage protein promoter, phaseolin promoter (Slightom et. al., *Proc. Natl. Acad. Sci. USA* 80:1897-901 (1983)), oleosin promoter (Plant et al., *Plant Mol. Biol.* 25:193-205 (1994); Rowley et. al., 1997, *Biochim. Biophys. Acta.* 1345:1-4 (1997); U.S. Patent No. 10 5,650,554; PCT WO 93/20216), zein promoter, glutelin promoter, starch synthase promoter, and starch branching enzyme promoter.

Alternatively, for some constructs it may be preferable to express the transgene only in the leaf. A suitable promoter for this purpose would include the C4PPDK promoter preceded by the 35S enhancer (Sheen, J. 15 *EMBO*, 12:3497-505 (1993)) or any other promoter that is specific for expression in the leaf.

Targeting Sequences

The 5' end of the transgene may be engineered to include sequences encoding plastid or other subcellular organelle targeting peptides linked in-frame with the transgene. A chloroplast targeting sequence is any peptide sequence that can target a protein to the chloroplasts or plastids, such as the transit peptide of the small subunit of the alfalfa ribulose-biphosphate carboxylase (Khoudi, et al., *Gene* 197:343-51 (1997)). A peroxisomal targeting sequence refers to any peptide sequence, either N-terminal, 20 internal, or C-terminal, that can target a protein to the peroxisomes, such as the plant C-terminal targeting tripeptide SKL (Banjoko & Trelease, *Plant Physiol.* 107:1201-08 (1995)).

SEQ ID NO:10 is the sequence of the chloroplast targeting signal from the alfalfa rubisco protein fused to the coding sequence of PhaC from 30 *Pseudomonas aeruginosa*. The underlined sequences “ccatgg”, “gcatgc”, and “gcggccgc” are Nco I, Sph I, and Not I restriction sites, respectively, introduced for cloning purposes. The Nco I site sequence contains the N-

terminal ATG of the targeting signal. The Sph I site fuses the ATG of the protein to be targeted to the N-terminal targeting signal. N-terminal methionines of the targeting signal and PhaC are indicated in bold.

- SEQ ID NO:11 is the sequence of the pea rubisco chloroplast targeting signal including 24 amino acids of the pea rubisco protein fused to the coding sequence of PhaG. The underlined sequences “ggatcc” and “tctaga” are BamHI and Xba I restriction sites, respectively, introduced for cloning purposes. N-terminal methionines of the targeting signal and PhaG are indicated in bold.
- SEQ ID NO:12 is the sequence of the chloroplast targeting signal from the alfalfa rubisco protein fused to the coding sequence of the reductase from *Alcaligenes eutrophus*. The underlined sequences “ccatgg”, “gcatgc”, and “gcggccgc” are Nco I, Sph I, and Not I restriction sites, respectively, introduced for cloning purposes. The Nco I site sequence contains the N-terminal ATG of the targeting signal. The Sph I site fuses the ATG of the protein to be targeted to the N-terminal targeting signal. N-terminal methionines of the targeting signal and PhaC are indicated in bold.

SEQ ID NO:13 is the sequence of the pea rubisco chloroplast targeting signal including 24 amino acids of the pea rubisco protein fused to the coding sequence of the synthase from *A. eutrophus*. The underlined sequences “ggatcc”, “tctaga”, are BamHI and Xba I restriction sites, respectively, introduced for cloning purposes. N-terminal methionines of the targeting signal and PhaG are indicated in bold.

#### Internal Ribosome Entry Sites (IRES)

In the following examples, an IRES pertains to any sequence that can initiate cap-independent translation of mRNA in plants, such as the IRES of the Tobamovirus (Ivanov, et al., *Virology* 232:32-43 (1997)), the IRES of the turnip mosaic potyvirus (Basso, et al., *J. General Virology*, 75:3157-65 (1994)), the IRES of the cow pea mosaic virus (Thomas, et al., *J. Virology*, 65:2953-59 (1991)), or the potato virus Y (Levis & Astier-Manifacier, *Virus Genes* 7:367-79 (1993)).

The sequence encoding the potato virus Y IRES ((SEQ ID NO:7); Karchi, et al. *Virus Genes* 4:215-24 (1990)) can be isolated from plasmid pTHC8 (American Type Culture Collection #45127) using primers PVY.c and PVY.r.

5

PVY.c            5'cgggatcccacaacataagaaaaacaacgcaaaaac            (SEQ ID NO:1)  
PVY.r            5'catgccatgggagtatgcttagtaatgaaggaaat            (SEQ ID NO:2)

The sequence encoding the IRES of the turnip mosaic potyvirus ((SEQ ID NO:8); Basso, et al., *J. Gen. Virol.* 75:3157-65 (1994)) can be isolated from plasmid pTUM1.4 (American Type Culture Collection #45107) using primers TMV.c and TMV.r.

10                TMV.c            5'cgggatccaaaatataaaaactcaacacaacata            (SEQ ID NO:3)  
15                TMV.r            5'catgccatgggttggtgattgcttgataacgacaa            (SEQ ID NO:4)

The sequence encoding the IRES of the cowpea mosaic virus ((SEQ ID NO:9); van Wezenbeek, et al., *EMBO J.* 2:941-46 (1983)) can be isolated from plasmid pTMB120 (American Type Culture Collection #45054) using 20 primers CMV.c and CMV.r.

CMV.c            5'cgggatccatgtttcttcactgaagcgaaatca            (SEQ ID NO:5)  
CMV.r            5'catgccatggcaaatttgggcagaatatacaga            (SEQ ID NO:6)

25                Poly Adenylation Signals

At the extreme 3' end of the transcript, a polyadenylation signal can be engineered. A polyadenylation signal refers to any sequence that can result in polyadenylation of the mRNA in the nucleus prior to export of the mRNA to the cytosol, such as the 3' region of nopaline synthase (Bevan, et 30 al., *Nucleic Acids Res.* 11:369-85 (1983)).

### Protein Encoding Sequences

The protein encoding sequences (also commonly referred to as “genes”, although in this context not typically including a promoter or other regulatory sequences) can encode any protein which is to be expressed.

5 In a preferred embodiment, the protein coding sequences encode enzymes required for the production of polyhydroxyalkanoate biopolymers. In another embodiment, the protein coding sequences encode different subunits of a single enzyme or multienzyme complex. Preferred two subunit enzymes include the two subunit PHA synthases. Preferred multi-enzyme 10 complexes include the fatty acid oxidation complexes. In another embodiment, the protein coding sequences encode proteins which impart insect and/or pest resistance to the plant. In the case of a protein coding for insect resistance, a *Bacillus thuringensis* toxin is preferred, in the case of a herbicide resistance gene, the coding sequence imparts resistance to 15 glyphosate, sulphosate or Liberty herbicides.

### *Marker Genes*

Selectable marker genes useful in practicing the methods described herein include proteins conferring antibiotic resistance, herbicide resistances, and detectable proteins such as the green fluorescent protein. Examples of 20 antibiotic resistance genes include the neomycin phosphotransferase gene nptII (U.S. Patent No. 5,034,322 and No. 5,530,196), hygromycin resistance gene (U.S. 5,668,298), and the bar gene encoding resistance to phosphinothricin (U.S. Patent No. 5,276,268). EP 0 530 129 A1 describes a positive selection system which enables the transformed plants to outgrow 25 the non-transformed lines by expressing a transgene encoding an enzyme that activates an inactive compound added to the growth media. U.S. Patent No. 5,767,378 describes the use of mannose or xylose for the positive selection of transgenic plants.

Representative screenable marker genes useful for practicing the methods 30 described herein include the β-glucuronidase gene (Jefferson et al., *EMBO J.* 6:3901-07 (1987); U.S. Patent No. 5,268,463) and native or modified green fluorescent protein gene (Cubitt et al., *Trends Biochem Sci.* 20:448-55

(1995); Pang et al., *Plant Physiol.* 112:893-900 (1996)). Some of these markers have the added advantage of introducing a trait, e.g., herbicide resistance, into the plant of interest, thereby providing an additional agronomic value on the input side.

5

### Enzymes required for Production of Polyhydroxyalkanoates

#### *Polyhydroxyalkanoates*

Several types of PHAs are known. It is useful to broadly divide the PHAs into two groups according to the length of their side chains and according to their pathways for biosynthesis. Those with short side chains, such as polyhydroxybutyrate (PHB), a homopolymer of R-3-hydroxybutyric acid units, are crystalline thermoplastics; PHAs with long side chains are more elastomeric. The former polymers have been known for about seventy years (Lemoigne & Roukhelman 1925), while the latter polymers are a relatively recent discovery (deSmet, et al., *J. Bacteriol.*, 154:870-78 (1983)).

10 Before this designation, however, PHAs of microbial origin containing both R-3-hydroxybutyric acid units and longer side chain units from C5 to C16 were identified (Wallen & Rowheder, *Environ. Sci. Technol.*, 8:576-79 (1974)). A number of bacteria which produce copolymers of D-3-hydroxybutyric acid and one or more long side chain hydroxyacid units

15 containing from five to sixteen carbon atoms have been identified more recently (Steinbuchel & Wiese, *Appl. Microbiol. Biotechnol.*, 37:691-97 (1992); Valentin et al., *Appl. Microbiol. Biotechnol.*, 36: 507-14 (1992); Valentin et al., *Appl. Microbiol. Biotechnol.*, 40:710-16 (1994); Abe et al., *Int. J. Biol. Macromol.*, 16:115-19 (1994); Lee et al., *Appl. Microbiol. Biotechnol.*, 42:901-09 (1995); Kato et al., *Appl. Microbiol. Biotechnol.*, 45:363-70 (1996); Valentin et al., *Appl. Microbiol. Biotechnol.*, 46:261-67 (1996); U.S. Patent No. 4,876,331 to Doi). Useful examples of specific two-component copolymers include PHB-co-3-hydroxyhexanoate (Brandl et al., *Int. J. Biol. Macromol.*, 11:49-55 (1989); Amos & McInerey, *Arch. Microbiol.*, 155:103-06 (1991); U.S. Patent No. 5,292,860 to Shiotani et al.).

20 Other representative PHAs are described in Steinbüchel & Valentin, *FEMS Microbiol. Lett.*, 128:219-28 (1995). Chemical synthetic methods have also

been applied to prepare racemic PHB copolymers of this type for applications testing (PCT WO 95/20614, PCT WO 95/20615, and PCT WO 96/20621).

Useful molecular weights of the polymers are between about 10,000  
5 and 4 million Daltons, and preferably between about 50,000 and 1.5 million Daltons. The PHAs preferably contain one or more units of the following formula:



wherein n is 0 or an integer; and

10 wherein R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, and R<sup>4</sup> are independently selected from saturated and unsaturated hydrocarbon radicals, halo- and hydroxy- substituted radicals, hydroxy radicals, halogen radicals, nitrogen-substituted radicals, oxygen-substituted radicals, and hydrogen atoms.

15 Monomeric units generally include hydroxybutyrate, hydroxyvalerate, hydroxyhexanoate, hydroxyheptanoate, hydroxyoctanoate, hydroxynonanoate, hydroxydecanoate, hydroxyundecanoate, and hydroxydodecanoate units. PHAs can include monomers and polymers and derivatives of 3-hydroxyacids, 4-hydroxyacids and 5-hydroxyacids.

#### *Enzymes for Polymer Production*

20 The following is a general description of enzymes useful for polymer production in a plant.

ACP-CoA transacylase refers to an enzyme capable of converting β-hydroxy-acyl ACPs to β-hydroxy-acyl CoAs, such as *phaG* encoded protein from *Pseudomonas putida* (Rehm, et al., *J. Biol. Chem.* 273:24044-51 25 (1998)). PHA synthase refers to a gene encoding an enzyme that polymerizes hydroxyacyl CoA monomer units to form polymer. Examples of PHA synthases include a synthase with medium chain length substrate specificity, such as *phaC1* from *Pseudomonas oleovorans* (Peoples & Sinskey, PCT WO 91/00917; Huisman, et al., *J. Biol. Chem.* 266:2191-98 30 (1991)) or *Pseudomonas aeruginosa* (Timm & Steinbuchel, *Eur. J. Biochem.* 209:15-30 (1992)) or the PHB synthase from *Alcaligenes eutrophus* with short chain length specificity (Peoples & Sinskey *J. Biol. Chem.* 264:15298-

303 (1989)). A range of PHA synthase genes and genes encoding enzymes involved in additional steps in PHA biosynthesis are described in Madison & Huisman, *Microbiology and Molecular Biology Reviews* 63:21-53 (1999)).

An  $\alpha$  subunit of  $\beta$ -oxidation enzyme complex refers to a  
5 multifunctional enzyme that minimally possesses hydratase and dehydrogenase activities (Figure 2). The subunit may also possess epimerase and  $\Delta$ 3-cis,  $\Delta$ 2-trans isomerase activities. Examples of  $\alpha$  subunits of  $\beta$ -oxidation are FadB from *E. coli* (DiRusso, *J. Bacteriol.* 172:6459-68 (1990)), FaoA from *Pseudomonas fragi* (Sato, et al., *J. Biochem.* 111:8-15 (1992)), and the *E. coli* open reading frame f714 that contains homology to multifunctional  $\alpha$  subunits of  $\beta$ -oxidation (Genbank Accession # 1788682).

A  $\beta$  subunit of  $\beta$ -oxidation enzyme complex refers to a polypeptide capable of forming a multifunctional enzyme complex with its partner  $\alpha$  subunit. The  $\beta$  subunit possesses thiolase activity (Figure 2). Examples of  $\beta$  subunits are FadA from *E. coli* (DiRusso, *J. Bacteriol.* 172:6459-68 (1990)), FaoB from *Pseudomonas fragi* (Sato, et al., *J. Biochem.* 111:8-15 (1992)), and the *E. coli* open reading frame f436 that contains homology to  $\alpha$  subunits of  $\beta$ -oxidation (Genbank Accession # AE000322; gene b2342).

A reductase refers to an enzyme that can reduce  $\beta$ -ketoacyl CoAs to  
20 R-3-OH-acyl CoAs, such as the NADH dependent reductase from *Chromatium vinosum* (Liebergesell & Steinbuchel, *Eur. J. Biochem.* 209:135-50 (1992)), the NADPH dependent reductase from *Alcaligenes eutropus* (Peoples & Sinskey *J. Biol. Chem.* 264:15293-97 (1989)), or the NADPH reductase from *Zoogleola ramigera* (Peoples, et al., *J. Biol. Chem.* 262:97-102 (1987); Peoples & Sinskey *J. Molecular Microbiol.* 3:349-57 (1989)).

A  $\beta$ -ketothiolase refers to an enzyme that can catalyze the conversion of acetyl CoA and an acyl CoA to a  $\beta$ -ketoacyl CoA, a reaction that is reversible (Figure 2). An example of such a thiolase is PhaA from  
30 *Alcaligenes eutropus* (Peoples & Sinskey *J. Biol. Chem.* 1989, 264:15293-97).

An acyl CoA oxidase refers to an enzyme capable of converting saturated acyl CoAs to  $\Delta 2$  unsaturated acyl CoAs (Figure 2). Examples of acyl CoA oxidases are POX1 from *Saccharomyces cerevisiae* (Dmochowska, et al., *Gene* 88:247-52 (1990)) and ACX1 from *Arabidopsis thaliana* (Genbank Accession # AF057044).

5 A catalase refers to an enzyme capable of converting hydrogen peroxide to hydrogen and oxygen. Examples of catalases are KatB from *Pseudomonas aeruginosa* (Brown, et al., *J. Bacteriol.* 177:6536-44 (1995)) and KatG from *E. coli* (Triggs-Raine & Loewen, *Gene* 52:121-28 (1987)).

10 Multi-step enzyme pathways are known for the biosynthesis of PHA copolymers from normal cellular metabolites and are particularly suited to the methods and constructs described herein. For example, pathways for incorporation of 3-hydroxyvalerate are described in PCT WO 98/00557. Pathways for incorporation of 4-hydroxybutyrate are described in PCT WO 15 98/36078 and PCT WO 99/14313.

### Plants

The transformation of suitable agronomic plant hosts using these vectors can be accomplished by a range of methods and plant tissues. Representative plants suitable for carrying out the methods include the 20 *Brassica* family including *napus*, *rappa*, *sp. carinata* and *juncea*; maize; soybean; cottonseed; sunflower; palm; coconut; safflower; peanut; mustards including *Sinapis alba*; and flax. Representative tissues which are suitable for transformation using these vectors include protoplasts, cells, callus tissue, leaf discs, pollen, and meristems.

25 **II. Methods**

#### Transformation Processes

Examples of suitable transformation procedures include 30 *Agrobacterium*-mediated transformation, biolistics, microinjection, electroporation, polyethylene glycol-mediated protoplast transformation, liposome-mediated transformation, and silicon fiber-mediated transformation (U.S. Patent No. 5,464,765; Gene Transfer to Plants, Potrykus & Spangenberg, eds., Springer-Verlag Berlin Heidelberg New York (1995);

Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins, Owen & Pen, eds., John Wiley & Sons Ltd. England (1996); and Methods in Plant Molecular Biology-a laboratory course manual, Maliga, et al., eds., Cold Spring Laboratory Press, New York (1995).

5 In order to generate transgenic plants using the constructs of the present invention, following transformation by any one of the methods described above, the following procedures can be used to obtain a transformed plant expressing the transgenes: select the plant cells that have been transformed on a selective medium; regenerate the plant cells that have  
10 been transformed to produce differentiated plants; select transformed plants expressing the transgene at such that the level of desired polypeptides is obtained in the desired tissue and cellular location.

For the specific crops useful for practicing the described invention, transformation procedures have been established (Gene Transfer to Plants,  
15 Potrykus & Spangenberg, eds., Springer-Verlag Berlin Heidelberg New York (1995); Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins, Owen & Pen, eds., John Wiley & Sons Ltd. England (1996); and Methods in Plant Molecular Biology-a laboratory course manual, Maliga, et al., eds., Cold Spring Laboratory Press, New York  
20 (1995)).

*Brassica napus* can be transformed as described, for example, in U.S. Patents No. 5,188,958 and No. 5,463,174. Other *Brassica* such as *rappa*, *carinata* and *juncea*, as well as *Sinapis alba*, can be transformed as described in Moloney et al., *Plant Cell Reports* 8:238-42 (1989)). Soybean can be  
25 transformed by a number of procedures, such as those described in U.S. Patents No. 5,015,580; No. 5,015,944; No. 5,024,944; No. 5,322,783; No. 5,416,011; and No. 5,169,770).

A number of transformation procedures have been reported for the production of transgenic maize plants including pollen transformation (U.S.  
30 Patent No. 5,629,183), silicon fiber-mediated transformation (U.S. Patent No. 5,464,765), electroporation of protoplasts (U.S. Patents No. 5,231,019; No. 5,472,869; and No. 5,384,253), gene gun (U.S. Patents No. 5,538,877

and No. 5,538,880), and *Agrobacterium*-mediated transformation (EP 0 604 662 A1; PCT WO 94/00977). The *Agrobacterium*-mediated procedure is particularly preferred, as single integration events of the transgene constructs are more readily obtained using this procedure which greatly facilitates  
5 subsequent plant breeding.

Cotton can be transformed by particle bombardment (U.S. Patents No. 5,004,863 and No. 5,159,135). Sunflower can be transformed using a combination of particle bombardment and *Agrobacterium* infection (EP 0 486 233 A2; U.S. Patent No. 5,030,572). Flax can be transformed by either  
10 particle bombardment or *Agrobacterium*-mediated transformation.

Recombinase technologies which are useful in practicing the methods described herein include the *cre-lox*, FLP/FRT and Gin systems. Methods by which these technologies can be used for the purposes described herein are described, for example, in U.S. Patent No. 5,527,695; Dale & Ow, *Proc. Natl. Acad. Sci. USA* 88:10558-62 (1991); and Medberry et al., *Nucleic Acids Res.* 23:485-90 (1995).

#### Producing Plants Containing Multiple Stacked Input Traits

The production of a plant that is tolerant to the herbicide glyphosate and that produces the *Bacillus thuringiensis* (BT) toxin can be used to illustrate the usefulness of poly-cistronic vectors for the creation of plants with stacked input traits. Glyphosate is a herbicide that prevents the production of aromatic amino acids in plants by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase). The overexpression of EPSP synthase in a crop of interest allows the application of glyphosate as a weed killer without killing the genetically engineered plant (Suh, et al., *Plant Mol. Biol.* 22:195-205 (1993)). BT toxin is a protein that is lethal to many insects, providing the plant that produces it protection against pests (Barton, et al., *Plant Physiol.* 85:1103-09 (1987)). Combining the two traits into one plant using a polycistronic IRES containing vector enables the production of stacked traits in a single transformation event, a major improvement over current art.  
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25  
30

The compositions and methods described herein will be further understood with reference to the following non-limiting examples.

**Example 1: IRES Containing Bicistronic Constructs**

The *in vivo* expression of two proteins from an IRES containing bicistronic construct with only one promoter and one polyadenylation signal can be demonstrated in protoplast transient expression assays.

5           A.     Preparation of Constructs

A suitable construct contains the following genetic elements (see Figure 3): a promoter active in leaves such as the 35S-C4PPDK light inducible plant promoter (Sheen, *J. EMBO*, 12:3497-505 (1993)); a gene encoding β-glucuronidase (GUS) (Jefferson, et al., *EMBO J.* 6:3901-07 (1987)); an IRES; a gene encoding an enhanced green fluorescent protein (EGFP; Clontech, Palo Alto, CA); and a polyadenylation signal.

To demonstrate the functionality of various IRES sequences in promoting translation of the second coding region in bicistronic constructs, plasmid pC4PPDK-GUS-TEV-EGFP was constructed. This construct allows the easy replacement of the tobacco etch virus (TEV) enhancer (Carrington & Freed, *J. Virology* 1990, 64:1590-97), cloned between genes encoding GUS and EGFP, with any IRES sequence flanked by a 5' BamHI and a 3' Nco I restriction site. Plasmid pC4PPDK-GUS-TEV-EGFP is unable to express GFP since the TEV enhancer is not able to promote cap-independent translation of the second gene in the bicistronic construct. Replacement of the TEV enhancer with a functional IRES upstream of the EGFP gene allows GFP expression.

25           Plasmid pC4PPDK-GUS-TEV-EGFP was constructed using the following multi-step procedure. A fragment containing the 35S-C4PPDK promoter was excised from plasmid 35S-C4PPDK-GFP (Sheen et al., *Plant Journal*, 8:777-84 (1995)) as an Xhol/BamHI fragment and inserted into the equivalent sites of plasmid pIRES2.EGFP (Clontech, Palo Alto, California) 30 forming plasmid pIRES2/C4PPDK. Plasmid pIRES2/C4PPDK contains the 35S-C4PPDK promoter inserted in front of the IRES from the

encephalomyocarditis virus (EMCV), the EGFP protein, and the polyadenylation signal of the bovine growth hormone.

A fragment containing the 35S-C4PPDK promoter, the EMCV IRES, and the EGFP gene was released from plasmid pIRES2/C4PPDK by digestion with Xho I/Not I and cloned into the Xho I/Not I sites of 35S-C4PPDK-GFP forming plasmid pUC18/C4PPDK-EMCVIRES-EGFP-nos. Plasmid pUC18/C4PPDK-EMCVIRES-EGFP-nos contains the 35S-C4PPDK promoter, the EMCV IRES, and the EGFP gene in front of the plant polyadenylation signal of plasmid 35S-C4PPDK-GFP.

10 The EMCV-IRES of plasmid pUC18/C4PPDK-EMCVIRES-EGFP-nos was replaced with a fragment encoding the TEV enhancer (Carrington & Freed) by digesting plasmid pUC18/C4PPDK-EMCVIRES-EGFP-nos with BamHI and Nco I. The TEV enhancer was ligated into the previously prepared vector DNA forming pC4PPDK-TEV-EGFP. Plasmid pC4PPDK-15 TEV-EGFP contains the C4PPDK promoter, the TEV enhancer, the EGFP gene, and a plant polyadenylation signal.

15 A fragment encoding GUS was ligated into the BamHI site of pC4PPDK-TEV-EGFP as follows. Plasmid pBI101.2 (Clontech, Palo Alto, CA), containing GUS flanked by SacI and BamHI sites, was digested with 20 SacI and the protruding ends were filled in with Klenow. BamHI linkers were attached to the blunt-ended fragments with T4 ligase. The sample was digested with BamHI resulting in the formation of a GUS fragment flanked by BamHI restriction sites. The GUS gene was cloned into the BamHI site of plasmid pC4PPDK-TEV-EGFP forming plasmid pC4PPDK-GUS-TEV-25 EGFP.

IRES sequences can be tested for functionality *in vivo* in plants by removing the TEV enhancer with BamHI and Nco I and inserting the sequence to be tested as a BamHI/Nco I fragment. EGFP, the second coding region in the bicistronic construct, requires the insertion of a functional IRES 30 immediately upstream in order for expression of EGFP to occur.

B. Expression of the GUS and EGFP Genes

Expression of the GUS and EGFP genes in IRES containing bicistronic constructs were tested using the following protoplast transient expression procedure. Two well-expanded leaves from 4-6 week old plants of *Arabidopsis thaliana* were harvested and the leaves were cut perpendicularly, with respect to the length of the leaf, into the smallest strips possible. The cut leaves were transferred to 20 mL of a solution containing 0.4 M mannitol and 10 mM MES, pH 5.7, in a 250 mL side armed flask. Additional leaves were cut such that the total number of leaves processed was between 50 and 60. After all leaves were cut, the solution in the flask was removed with a pipette and 20 mL of a cellulase/macerozyme solution was added. The enzyme solution was prepared as follows: 8.6 mL of H<sub>2</sub>O, 10 mL of 0.8 M mannitol, and 400 µL 0.5M MES, pH 5.7, were mixed and heated to 55°C. R-10 cellulase (0.3 g, Serva) and R-10 macerozyme (0.08 g, Serva) was added and the solution was mixed by inversion. The enzyme solution was incubated at room temperature for 10-15 min. A 400 µL aliquot of 1M KCl and 600 µL of 1M CaCl<sub>2</sub> were added to the enzyme solution, mixed, and the resulting solution was sterile filtered through a 0.2 µM filter. After addition of the enzyme solution, the flask was swirled gently to mix the leaf pieces and a house vacuum was applied for 5 minutes. Prior to releasing the vacuum, the flask was swirled gently to release air bubbles from the leaf cuts. The leaves were digested for 2-3 hours at room temperature.

Protoplasts were released from the leaves by gently swirling the flask for 1 min and filtering the protoplast containing solution was filtered through 25 nylon mesh (62 µM mesh). The eluent was transferred to a sterile, screw top, 40 mL conical glass centrifuge tube and centrifuged at 115 g for 2 min. The supernatant was removed with a Pasteur pipette and 10 mL ice cold W5 solution was added (W5 solution containing 154 mM NaCl, 125 mM CaCl<sub>2</sub>, 5 mM KCl, 5 mM glucose, and 1.5 mM MES, pH 5.7). The sample was 30 mixed by rocking the tube end over end until all of the pellet was in solution. The sample was centrifuged as described above and the supernatant was removed with a Pasteur pipette. The protoplast pellet was resuspended in 5

mL of ice cold W5. The sample was incubated for 30 minutes on ice so that the protoplasts became competent for transformation. Intact protoplasts were quantitated using a hemacytometer. The protoplasts were isolated by centrifugation and resuspended in an ice cold solution containing 0.4 M 5 mannitol, 15 mM MgCl<sub>2</sub>, and 5 mM MES, pH 5.7, to approximately 2 x 10<sup>6</sup> protoplasts/mL.

Plasmid DNA samples (160 µg, 1 µg/µL stock) for transformation were placed in 40 mL glass conical centrifuge tubes. An aliquot of 10 protoplasts (800 µL) was added to an individual tube followed immediately by 800 µL of a solution containing 40% PEG 3350 (w/v), 0.4 M mannitol, and 100 mM Ca (NO<sub>3</sub>)<sub>2</sub>. The sample was mixed by gentle inversion and the procedure repeated for remaining samples.

All transformation tubes were incubated at room temperature for 30 minutes. Protoplasts samples were diluted sequentially with 1.6 mL, 4 mL, 8 15 mL, and 12 mL of W5 solution. Between each dilution step, the sample was gently mixed by inversion and incubated at room temperature for 5 minutes. Protoplasts were harvested by centrifugation (115 g) and the supernatant was carefully removed with a Pasteur pipette. Protoplasts were resuspended in 4 mL of a solution containing 0.5 M mannitol, 5 mM MES, pH 5.7, 20 mM 20 KCl, and 5 mM CaCl<sub>2</sub>. For transient expression of the transformed DNA, the samples were incubated at room temperature for 16 hours under a 20 Watt table top plant light.

GUS activity in protoplasts can be measured as described, for example, in Jefferson, *Plant Mol. Biol. Rep.* 5:387-405 (1987). For detection 25 of GFP expression, *Arabidopsis* protoplast samples can be analyzed by fluorescent microscopy (e.g., Sheen, et al., *Plant J.*, 8:777-84 (1995)). Protoplasts expressing GFP fluoresce under green light such that transformed cells appear green. Untransformed cells are red due to the autofluorescence of the chloroplasts. Alternatively, GFP expression can be analyzed by 30 Western detection of the protein.

Samples from transient expression experiments were prepared for Western analysis as follows. Protoplasts were harvested by centrifugation

(115 g) and the supernatant removed. An aliquot (14 µL) of 7X stock of protease inhibitor stock was added to the sample and the sample brought to a final volume of 100 µL with a solution containing 0.5 M mannitol, 5 mM MES, pH 5.7, 20 mM KCl, 5 mM CaCl<sub>2</sub>. The 7X stock of protease inhibitors was prepared by dissolving one “Complete Mini Protease Inhibitor Tablet” (Boehringer Manneheim) in 1.5 mL 0.5 M mannitol, 5 mM MES, pH 5.7, 20 mM KCl, 5 mM CaCl<sub>2</sub>. The protoplasts were disrupted in a 1.5 mL centrifuge tube using a pellet pestle mixer (Kontes) for 30 s. Soluble proteins were separated from insoluble proteins by centrifugation at maximum speed in a microcentrifuge (10 min, 4 °C). The protein concentration of the soluble fraction was quantitated using the Bradford dye-binding procedure with bovine serum albumin as a standard (Bradford, *Anal. Biochem.* 1976, 72:248-54). The insoluble protein was resuspended in 100 µL 1X gel loading buffer (New England Biolabs, Beverly, MA) and a volume equal to that loaded for the soluble fraction was prepared for analysis. Samples from the soluble and insoluble fractions of the protoplast transient expression experiment, as well as standards of green fluorescent protein (Clontech, Palo Alto, CA), were resolved by SDS-PAGE and proteins were blotted onto PVDF. Detection of transiently expressed proteins was performed by Western analysis using Living Colors Peptide Antibody to GFP (Clontech, Palo Alto, CA) and the Immun-Star Chemiluminescent Protein Detection System (BioRad, Hercules, CA).

The results demonstrated that the proteins were expressed.

#### **Example 2: Transformation of Tobacco Plants**

In Pseudomonads of the RNA I homology group, PHAs consisting of saturated medium-chain-length 3-hydroxyalkanoic acids are synthesized from monomer units that are thought to be derived from acyl carrier protein (ACP) intermediates of de novo fatty acid biosynthesis (Eggink, et al., *FEMS Microbiol. Rev.* 1992, 103:159-64; Saito & Doi, *Int. J. Biol. Macromol.* 1993, 15:287-92; Huijberts, et al., *J. Bacteriol.* 1994, 176:1661-66). A transacylase that is capable of converting acyl ACPS to acyl CoAs has recently been cloned (Rehm, et al., *J. Biol. Chem.* 1998, 273:24044-51).

Targeting of both the transacylase and a medium chain length synthase to the chloroplasts of a plant, the site of fatty acid biosynthesis, should divert 3-hydroxyacyl ACPS from fatty acid biosynthesis to polymer (Figure 7). Expression of all constructs can be targeted to the leaves, using a leaf specific promoter, or the seeds, using a seed specific promoter.

The genes encoding PhaC and PhaG can be inserted into a plant in a single transformation event using a multi-gene construct where each gene is flanked by its own promoter or polyadenylation signal. A suitable construct for this purpose would include the following genetic elements (Figure 8): a leaf specific promoter, a chloroplast targeting signal, the coding sequence of PhaG from *Pseudomonas putida*, a polyadenylation sequence, a leaf specific promoter, a chloroplast targeting signal, the coding sequence of a medium chain length PhaC, and a polyadenylation sequence. The construct can be transformed into a plant, such as tobacco, for PHA production in leaves.

Alternatively, a construct containing only one promoter, one polyadenylation signal, and an IRES can be constructed. A suitable construct for this purpose would include the following genetic elements (Figure 9a): a leaf specific promoter, a chloroplast targeting signal fused to a gene encoding a ACP-CoA transacylase, an IRES sequence, a chloroplast targeting signal fused to a gene encoding a PHA synthase that accepts medium chain length substrates, and a polyadenylation signal at the extreme 3' end of the cassette. The construct can be transformed into a plant, such as tobacco, for PHA production in leaves.

Constructs were transformed into tobacco using the following procedure. In a laminar flowhood under aseptic conditions, leaves from a tobacco plant were sterilized for 15 minutes in a one liter beaker containing a solution of 10% bleach and 0.1% Tween 20. The sterilized leaves were washed in one liter of water for 10 minutes, the water decanted, and the washing step repeated two additional times. The intact part of the leaves were cut in small pieces with a scalpel, avoiding any injured areas of the leaves. An aliquot (20 mL) of MS-suspension was mixed with 5 mL of an overnight culture of *Agrobacterium*, carrying the construct to be transformed

[MS-suspension contains (per L) 4.3 g MS salts, 1 mL of B5 vitamins (Sigma, St. Louis, MO), 30 g sucrose, 2 mg *p*-chlorophenoxyacetic acid, and 0.05 mg kinetin, pH 5.8]. The tobacco leaf pieces were introduced into the solution and vortexed for a few seconds. The leaves were removed, wiped 5 on sterile filter paper, and placed in a petri dish to remove the excess *Agrobacterium* solution. An aliquot (1 mL) of tobacco cell culture was added on top of solidified MS-104 medium in a petri dish and a sterile piece of filter paper was placed directly on the top of the culture [MS-104 medium contains (per L) 4.3 g MS salts, pH 5.8, 1 mL B5 vitamins, 30 g sucrose, 1 10 mg benzylaminopurine, 0.1 mg naphthalene acetic acid, and 8 g of phytagar]. The tobacco leaf pieces were placed on top of the filter and incubated for two days at 25 °C. The leaf pieces were transferred, face-up, to a petri dish containing MS-selection medium and gently pressed into the medium [MS-selection medium contains (per L) 4.3 g MS salts, pH 5.8, 1 mL B5 vitamins, 15 30 g sucrose, 1 mg benzylaminopurine, 0.1 mg naphthalene acetic acid, 500 mg of carbenicillin, appropriate drug for selection of resistance of transformation vector, and 6.5 g of phytagar]. The dishes were wrapped with parafilm and incubated at 25 °C for 3 weeks. The leaves were transferred to fresh MS-selection medium and incubation at 25 °C is continued until 20 plantlets appear. Plantlets were separated from the callus and placed in test-tubes (24 x 3 cm) containing 10 mL of MS-rooting medium [MS-rooting medium contains (per L) 4.3 g MS salts, pH 5.8, 1 mL B5 vitamins, 30 g sucrose, and 6.5 g of phytagar]. When roots were 1 cm in length, the transformed plants were transferred to soil and covered with an inverted, 25 transparent, plastic cup in which a hole had been pierced in the bottom. After 4 or 5 days, the cup was removed and transformed tobacco plants were grown under standard conditions.

The results demonstrated expression in the chloroplasts of the leaves.

### **Example 3: PHA Formation in the Peroxisomes of Plant Leaves**

30 Pseudomonads, such as *Pseudomonas oleovorans*, are also capable of producing medium chain length polymers when grown in the presence of fatty acids (Lageveen et al., *Appl. & Environ. Microbiol.* 54:2924-32 (1988);

Ramsay, et al., *Appl. & Environ. Microbiol.* 57:625-29 (1991)). The 3-hydroxyacyl CoA monomer units are derived from intermediates formed during degradation of the fatty acid to acetyl CoA using β-oxidation pathways (Figure 2).

5 Medium chain length PHAs can be formed from fatty acids in the peroxisomes of leaves. A suitable construct for this purpose (Figure 9b) contains a leaf specific promoter, a gene encoding a PHA synthase that accepts medium chain length substrates fused to a peroxisomal targeting signal, an IRES, a multifunctional α subunit of β-oxidation fused to a peroxisomal targeting signal, and a polyadenylation signal. Plants naturally contain α and β subunits of β-oxidation in the peroxisomes that convert fatty acyl CoAs to acetyl CoA. It has been demonstrated that targeting of a medium chain length synthase to the peroxisomes of *Arabidopsis thaliana* (Mittendorf, et al., *Proc. Natl. Acad. Sci. USA* 95:13397-402 (1998)) or 10 tobacco plants (Hahn, J.J., Ph.D. Thesis, University of Minnesota, Feb. 15 1998) produce a small amount of polymer. The expression of a transgene encoding an additional α subunit of β-oxidation increases the ratio of α subunits, containing activities capable of monomer formation, to β-subunits, containing a thiolase activity that will channel the substrate down the degradative pathway (Figure 2). This increased ratio may help to divert 15 more carbon from fatty acid degradation to PHA formation in the peroxisomes. The construct described in Figure 9b can be transformed into crops for the production of medium chain length polymer in the peroxisomes of leaves. For example, tobacco can be transformed as previously described.

20 **25 Example 4: Constructs Transformed into *Brassica napus***

The yields of polymer previously observed in *Arabidopsis* peroxisomes upon targeting a medium chain length synthase to the peroxisomes (Mittendorf, et al., *Proc. Natl. Acad. Sci. USA* 1998, 95:13397-402) may be low due to the rapid degradation of fatty acids to acetyl CoA, 30 preventing diversion of 3-hydroxyacyl CoAs to PHAs (Figure 2). Creation of an artificial β-oxidation pathway in the cytosol would allow the optimal enzymes to be chosen for both the degradation of fatty acids to the

appropriate chain length and for the diversion of carbon from the fatty acid degradation pathway to polymer formation. A suitable construct (Figure 9c) for this purpose includes a seed specific promoter, a gene encoding acyl CoA oxidase in which the peroxisomal targeting signal has been removed, an  
5 IRES, a gene encoding a medium chain length synthase, an IRES, a gene encoding an  $\alpha$  subunit of  $\beta$ -oxidation, an IRES, a gene encoding a  $\beta$  subunit of  $\beta$ -oxidation, and a polyadenylation signal.

The construct outlined in Figure 9c can be transformed into oil seed crops for PHA production within the seeds. For example, the constructs can  
10 be transformed into *Brassica napus* using the following procedure (Moloney et al., *Plant Cell*, 8:238-42 (1989)). Seeds of *Brassica napus* cv. Westar were surfaced sterilized in 10% commercial bleach (Javex, Colgate-Palmolive Canada Inc.) for 30 min. with gentle shaking. The seeds were washed three times in sterile distilled water. Seeds were placed on  
15 germination medium comprising Murashige-Skoog (MS) salts and vitamins, 3% sucrose and 0.7% phytagar, pH 5.8 at a density of 20 per plate and maintained at 24 °C in a 16 h light/8 h dark photoperiod at a light intensity of 60-80  $\mu\text{Em}^{-2}\text{s}^{-1}$  for 4-5 days. Constructs were introduced into *Agrobacterium tumefaciens* strain EHA101 (Hood et al, *J. Bacteriol.* 1986, 168:1291-301) by electroporation. Prior to transformation of cotyledonary petioles, single colonies of strain EHA101 harboring each construct were grown in 5 mL of minimal medium, supplemented with the appropriate selection antibiotics for the transformation vector, for 48 h at 28 °C. One mL of bacterial suspension was pelleted by centrifugation for 1 min in a  
20 microfuge. The pellet was resuspended in 1 mL minimal medium.  
25

For transformation, cotyledons were excised from 4 to 5 day old seedlings so that they included approximately 2 mm of petiole at the base. Individual cotyledons with the cut surface of their petioles were immersed in diluted bacterial suspension for 1 s and immediately embedded to a depth of  
30 approximately 2 mm in co-cultivation medium, MS medium with 3% sucrose and 0.7% phytagar, enriched with 20  $\mu\text{M}$  benzyladenine. The inoculated cotyledons were plated at a density of 10 per plate and incubated

under the same growth conditions for 48 h. After co-cultivation, the cotyledons were transferred to regeneration medium comprising MS medium supplemented with 3% sucrose, 20 µM benzyladenine, 0.7% phytagar, pH 5.8, 300 mg/L timentinin and the appropriate antibiotics for selection of the 5 plant transformation vector.

After 2-3 weeks, regenerant shoots were obtained, cut, and maintained on 'shoot elongation' medium (MS medium containing 3% sucrose, 300 mg/L timentin, 0.7% phytagar and the appropriate antibiotic) in Magenta jars. The elongated shoots were transferred to 'rooting' medium 10 comprising MS medium, 3% sucrose, 2 mg/L indole butyric acid, 0.7% phytagar and 500 mg/L carbenicillin. After the emergence of roots, plantlets were transferred to potting mix (Redi Earth, W.R. Grace & Co. Canada Ltd.). The plants were maintained in a misting chamber (75% relative humidity) under the same growth conditions.

15 The results demonstrate expression.

#### **Example 5: Cytosolic Production of Short Chain PHAs in Leaves**

Short chain PHAs can be produced by co-expressing enzymes that divert acetyl CoA from a plant's metabolism with a synthase that accepts short chain length substrates. β-ketothiolase and acetoacetyl CoA reductase 20 will convert acetyl CoA to R-3-hydroxybutyryl CoA, the substrate for PHA synthase. Since acetyl CoA is found in multiple tissues and organelles in the plant, a multitude of strategies can be developed for short chain production by using the appropriate organelle targeting signals and/or tissue specific promoters. For the cytosolic production of short chain PHAs in leaves, an 25 appropriate expression cassette includes the following (Figure 10a): a leaf specific promoter, a reductase, an IRES, a synthase that accepts short chain substrates, and a polyadenylation signal at the extreme 3' end of the cassette. PHB production has been demonstrated in the leaves of *Arabidopsis thaliana* by inserting transgenes expressing synthase and reductase while depending 30 on the endogenous thiolase in the cytoplasm for conversion of acetyl CoA to acetoacetyl CoA (Poirier, et al., *Science* 1992, 256:520-23). Insertion of a transgene expressing thiolase into the construct in Figure 10a may increase

the yield of PHB in the cytoplasm of the leaves. For this purpose, an additional IRES element as well as a nucleotide sequence encoding  $\beta$ -ketothiolase, can be inserted in the construct outlined in Figure 10a to form the construct outlined in Figure 10b. Both constructs can be transformed 5 into tobacco plants, as previously described, for production of short chain PHAs in tobacco leaves.

**Example 6: Cytosolic Production of Short Chain PHAs in Oilseeds**

Short chain PHAs can also be produced in the cytosol of oilseeds. An appropriate expression cassette includes the following (Figure 10a): a 10 seed specific promoter, a reductase, an IRES, a synthase that accepts short chain substrates, and a polyadenylation signal at the extreme 3' end of the cassette. Insertion of a transgene expressing thiolase may increase the yield of PHB in the cytoplasm of seeds. For this purpose, an additional IRES element, as well as a gene expressing  $\beta$ -ketothiolase, can be inserted in the 15 seed specific promoter construct outlined in Figure 10a to form the construct outlined in Figure 10b.

**Example 7: Short Chain PHA Production in the Chloroplasts of Leaves**

For short chain PHA production in the chloroplasts of leaves, an appropriate expression cassette includes the following (Figure 10c): a leaf 20 specific promoter, a chloroplast targeting signal fused to a reductase, an IRES, a chloroplast targeting signal fused to a synthase that accepts short chain substrates, and a polyadenylation signal at the extreme 3' end of the cassette. Insertion of a transgene expressing thiolase may increase the yield of PHB in the chloroplasts of the leaves. For this purpose, an additional 25 IRES element as well as a gene expressing  $\beta$ -ketothiolase fused to a chloroplast targeting signal, can be inserted into the construct outlined in Figure 10c to form the construct outlined in Figure 10d. Both constructs can be transformed into plants, such as tobacco, for production of short chain PHAs in the chloroplasts of leaves.

30 **Example 8: Short Chain PHA Production in the Plastids of Oilseeds**

For short chain PHA production in the plastids of oilseeds, an appropriate expression cassette includes the following (Figure 10c): a seed

specific promoter, a chloroplast targeting signal fused to a reductase, an IRES, a chloroplast targeting signal fused to a synthase that accepts short chain substrates, and a polyadenylation signal at the extreme 3' end of the cassette. Insertion of a transgene expressing thiolase may increase the yield  
5 of PHB in the plastids of seeds. For this purpose, an additional IRES element as well as a gene expressing  $\beta$ -ketothiolase fused to a chloroplast targeting signal, can be inserted in the construct outlined in Figure 10c to form the construct outlined in Figure 10d. The above constructs can be transformed into an oil seed crop, such *Brassica napus* whose transformation  
10 has been described above, for production of short chained PHAs in the plastids of oil seeds.

**Example 9: Short Chain PHA Production in the Peroxisomes of Leaves**

Short chain length PHAs can be formed in the peroxisomes of leaves by engineering enzymes that will capture the acetyl CoA as fatty acids are degraded by  $\beta$ -oxidation enzymes. An appropriate construct for targeting  
15 reductase and synthase to the peroxisomes of leaves would contain the following (Figure 10e): a leaf specific promoter, a gene encoding reductase fused to a peroxisomal targeting signal, an IRES sequence, a gene encoding a short chain synthase fused to a peroxisomal targeting signal, and a  
20 polyadenylation signal. The expression of an additional thiolase in the peroxisomes of seeds may serve to increase PHA yield. For this purpose, an additional IRES sequence, as well as a gene encoding a thiolase fused to a peroxisomal targeting signal, can be inserted in the construct outlined in Figure 10e forming the construct in Figure 10f.

**Example 10: Short Chain PHA Production from Fatty Acids**

Short chain PHA production from fatty acids can be achieved in the cytosol by engineering an artificial pathway for fatty acid  $\beta$ -oxidation (Figure 2). A suitable construct for this purpose contains the following  
25 (Figure 11a): a seed specific promoter, a gene encoding an acyl CoA oxidase, an IRES, a gene encoding a short chain length synthase, an IRES, a gene encoding an  $\alpha$ -subunit of  $\beta$ -oxidation, an IRES, a gene encoding a  $\beta$ -subunit of  $\beta$ -oxidation, an IRES, a gene encoding a reductase, and a

polyadenylation signal. The addition of a  $\beta$ -ketothiolase may allow more PHA to be formed. For this purpose, the construct outlined in Figure 11a can be modified to contain an additional IRES and a gene encoding a  $\beta$ -ketothiolase forming the construct outlined in Figure 11c.

5        Acyl CoA oxidases form hydrogen peroxide as a byproduct when converting acyl CoAs to  $\Delta 2$  unsaturated acyl CoAs. If hydrogen peroxide proves harmful to the plants, a gene encoding catalase can be inserted to convert the hydrogen peroxide to water and oxygen. For this purpose, the construct described in Figure 11a can be modified to contain an additional  
10      IRES sequence and a gene encoding a catalase, forming the construct outlined in Figure 11b.

The above constructs can be transformed into oil seed crops, such as *Brassica napus*, as previously described.

**Example 11: Glyphosate-Resistant Plant**

15      The construct described in Figure 12 can be used to create a plant that is resistant to glyphosate and produces BT toxin using a single transformation event. The construct contains a constitutive promoter that is active in all plant tissues, a gene encoding either wild-type enolpyruvylshikimate-3-phosphate synthase or a glyphosate resistant mutant of the enzyme (Stalker,  
20      et al., *J. Biol. Chem.* 1985, 260:4724-28), an IRES, a gene encoding the *Bacillus thuringiensis* endotoxin (Schnepp, et al., *J. Biol. Chem.* 1985, 260:6264-72; Schnepp, et al., *J. Biol. Chem.* 1985, 260:6273-80), and a polyadenylation signal. This construct can be transformed into any agronomically important crop to provide a plant that is herbicide resistant  
25      and that produces its own insecticide.

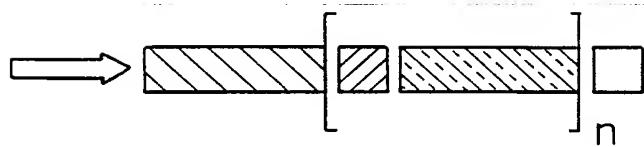
We claim:

1. An expression cassette comprising  
a single plant promoter at the 5'-end of the construct,  
a first protein encoding sequence 3' to the promoter,  
an internal ribosome entry site sequence 3' to the first protein  
encoding sequence,  
a second protein encoding sequence immediately 3' to the internal  
ribosome entry site, and  
a polyadenylation sequence 3' to the second protein encoding  
sequence.
2. The cassette of claim 1 wherein the promoter is selected from the  
group consisting of inducible, constitutive, and tissue specific plant  
promoters.
3. The cassette of claim 2 wherein the tissue specific plant promoter is  
selected from the group consisting of carboxylase promoter, 2S albumin  
promoter, seed storage protein promoter, phaseolin promoter, oleosin  
promoter, zein promoter, glutelin promoter, starch synthase promoter, starch  
branching enzyme promoter, and leaf expression specific promoters.
4. The cassette of claim 1 further comprising at least one nucleotide  
sequence 5' to at least one protein encoding sequence, the sequence encoding  
a peptide signal that targets the protein to a particular compartment of the  
cell.
5. The cassette of claim 4 wherein the compartment is selected from the  
group consisting of chloroplasts and plastids and peroxisomes.
6. The cassette of claim 1 wherein the protein encoding sequences  
encode at least one marker protein selected from the group consisting of  
proteins conferring antibiotic resistance, herbicide resistances, and detectable  
proteins.
7. The cassette of claim 1 further comprising a second internal ribosome  
entry site sequence and a third protein encoding sequence.

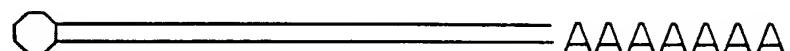
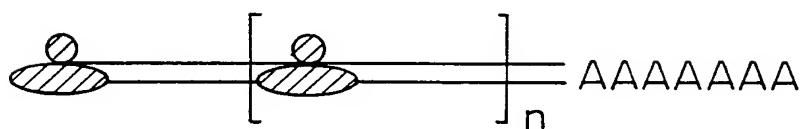
8. The cassette of claim 1 wherein the protein encoding sequences encode proteins selected from the group consisting of ACP-CoA transacylase, PHA synthase, an  $\alpha$  subunit of  $\beta$ -oxidation enzyme complex, a  $\beta$  subunit of  $\beta$ -oxidation enzyme complex, a reductase, a  $\beta$ -ketothiolase, an acyl CoA oxidase, and a catalase.
9. The cassette of claim 1 comprising a leaf specific promoter, a chloroplast targeting signal fused to a gene encoding a ACP-CoA transacylase, an internal ribosome entry site sequence, a chloroplast targeting signal fused to a gene encoding a PHA synthase that accepts medium chain length substrates, and a polyadenylation signal at the extreme 3' end of the cassette.
10. The cassette of claim 1 comprising a leaf specific promoter, a gene encoding a PHA synthase that accepts medium chain length substrates fused to a peroxisomal targeting signal, an internal ribosome entry site sequence, a multifunctional  $\alpha$  subunit of  $\beta$ -oxidation complex fused to a peroxisomal targeting signal, and a polyadenylation signal.
11. The cassette of claim 1 comprising a seed specific promoter, a gene encoding acyl CoA oxidase in which the peroxisomal targeting signal has been removed, an internal ribosome entry site sequence, a gene encoding a medium chain length synthase, an IRES, a gene encoding an  $\alpha$  subunit of  $\beta$ -oxidation, an IRES, a gene encoding a  $\beta$  subunit of  $\beta$ -oxidation, and a polyadenylation signal.
12. Plant material transformed with an expression cassette comprising a single plant promoter at the 5'-end of the construct, a first protein encoding sequence 3' to the promoter, an internal ribosome entry site 3' to the first protein encoding sequence, a second protein encoding sequence immediately 3' to the internal ribosome entry site sequence, and a polyadenylation sequence 3' to the second protein encoding sequence.

13. The plant material of claim 12 wherein the plants are selected from the group consisting of *Brassica*; maize; soybean; cottonseed; sunflower; palm; coconut; safflower; peanut; mustards; and flax.
14. The plant material of claim 9 wherein the material is tissue selected from the group consisting of protoplasts, cells, callus tissue, leaf discs, pollen, and meristems.
15. The plant material of claim 9 wherein the expression cassette is as defined by any of claims 2-11.
16. A method for expression of heterologous genes in plant material comprising transforming the material with an expression cassette comprising a single plant promoter at the 5'-end of the construct, a first protein encoding sequence 3' to the promoter, an internal ribosome entry site 3' to the first protein encoding sequence, a second protein encoding sequence immediately 3' to the internal ribosome entry site, and a polyadenylation sequence 3' to the second protein encoding sequence.
17. The method of claim 16 wherein the plant material is transformed using a method selected from the group consisting of *Agrobacterium*-mediated transformation, biolistics, microinjection, electroporation, polyethylene glycol-mediated protoplast transformation, liposome-mediated transformation, and silicon fiber-mediated transformation.
18. The method of claim 16 wherein the cassette is as defined by any of claims 2-11.

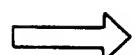
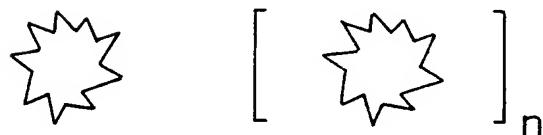
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**FIG. 1A**

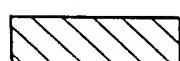
Transcription

**FIG. 1B****FIG. 1C**

Translation

**FIG. 1D**

promoter

3' non coding  
region

gene 1



ribosomes



IRES



proteins

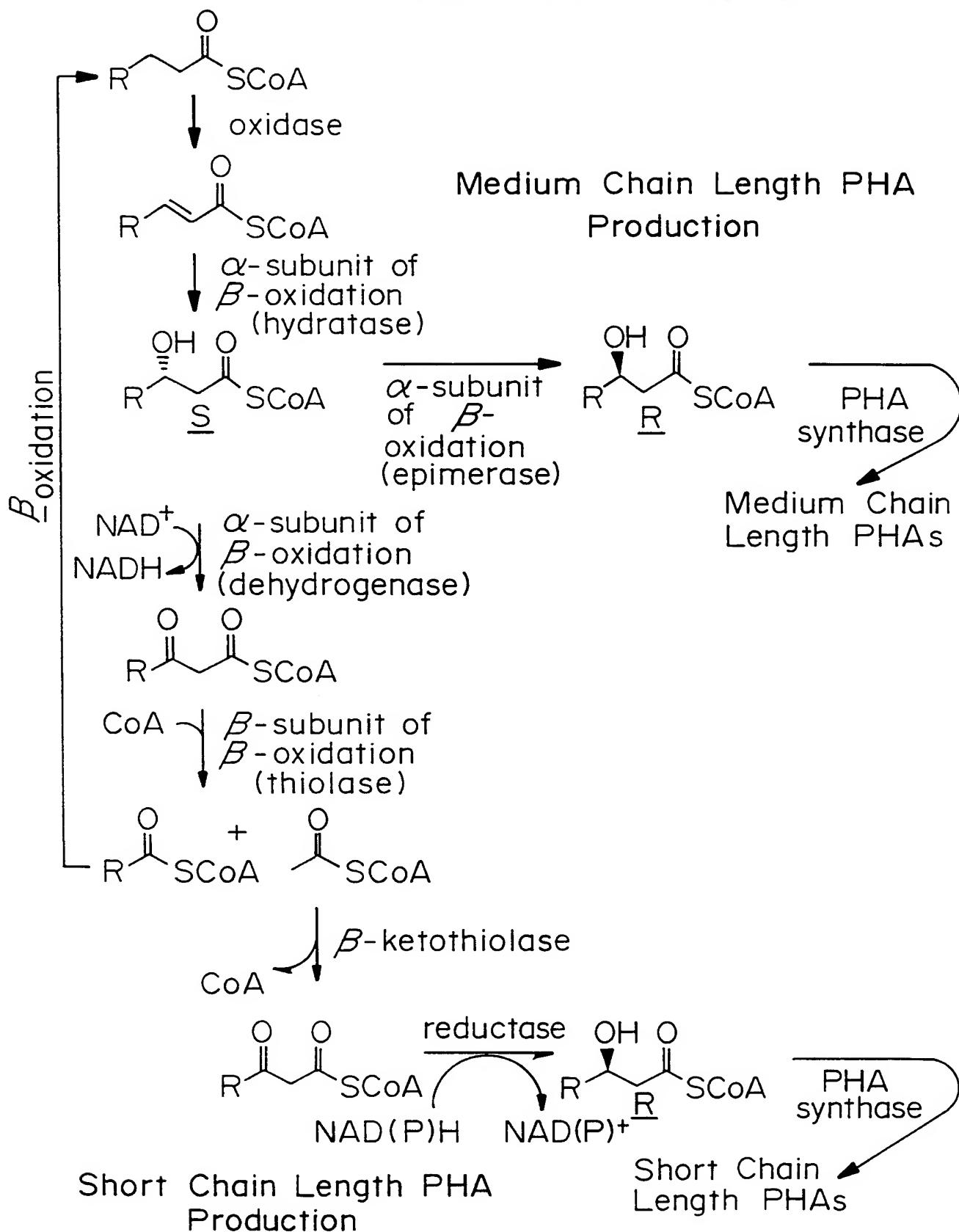


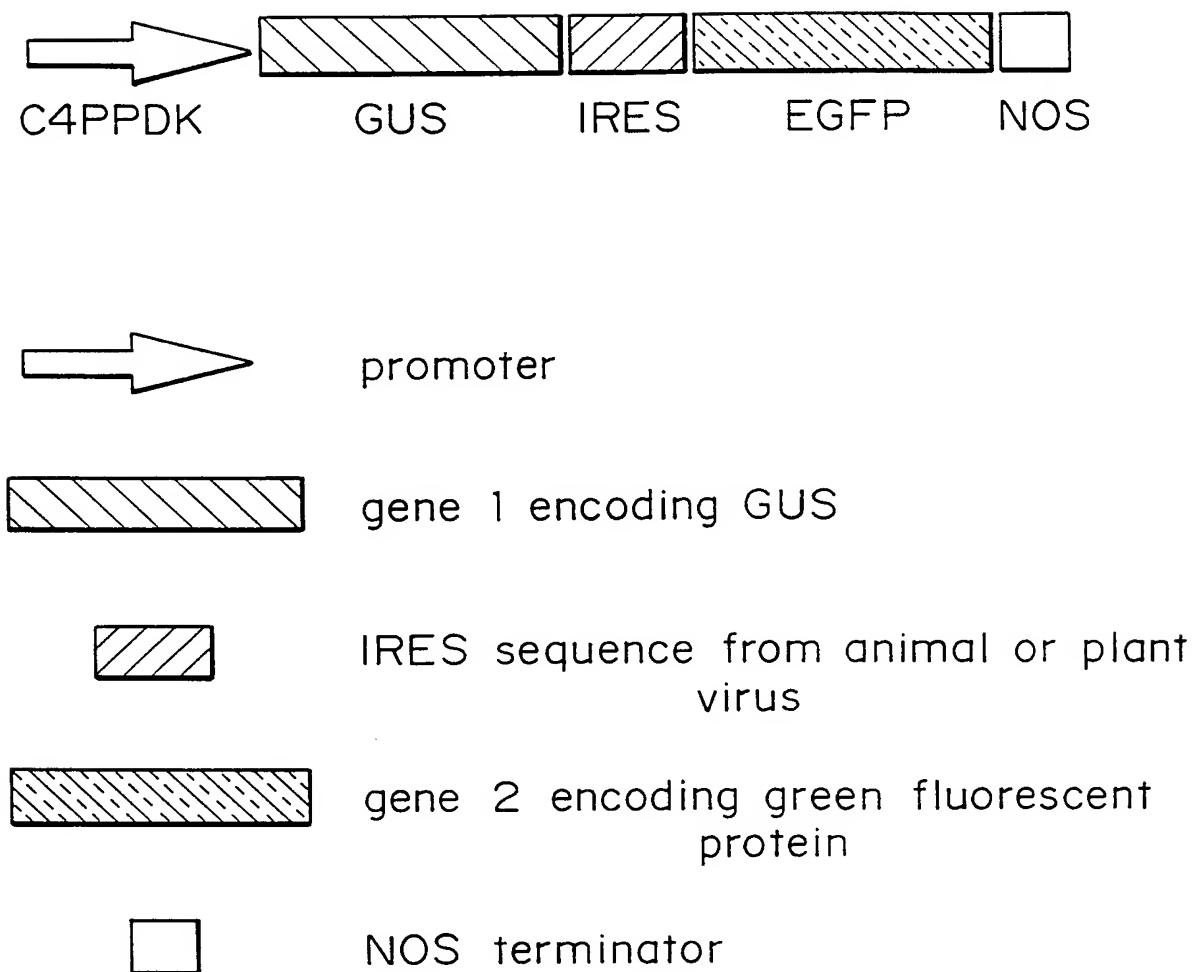
gene 2

AAAAAAA

poly A signal

2/9  
*FIG. 2*





***FIG. 3***

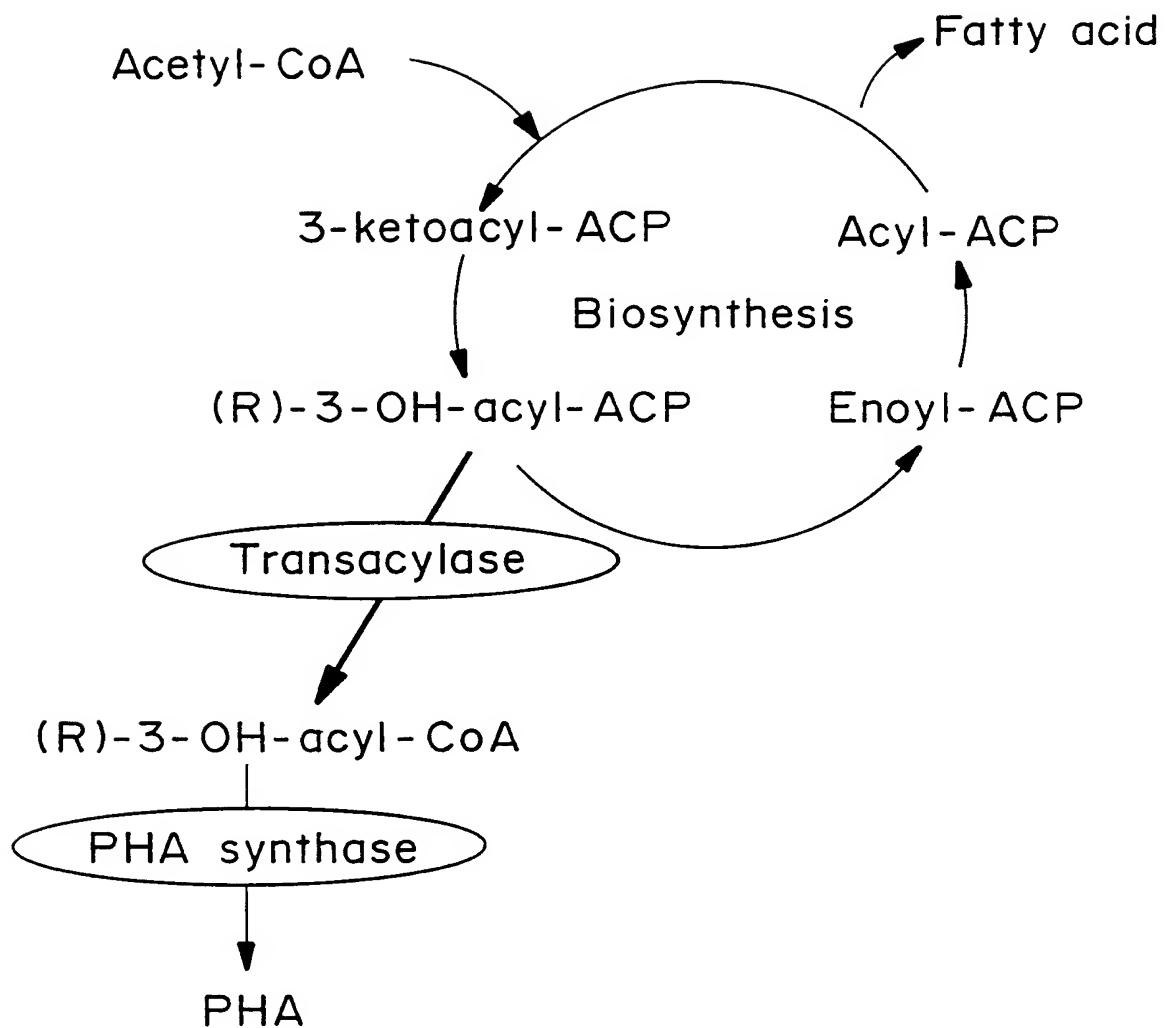
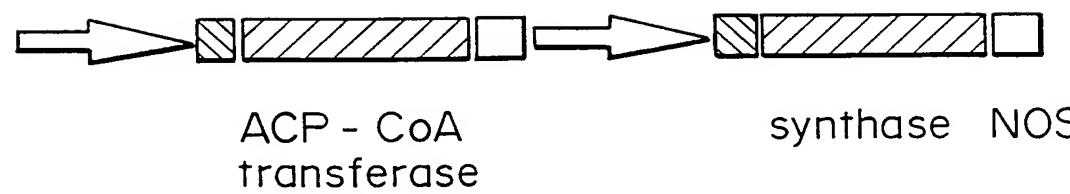
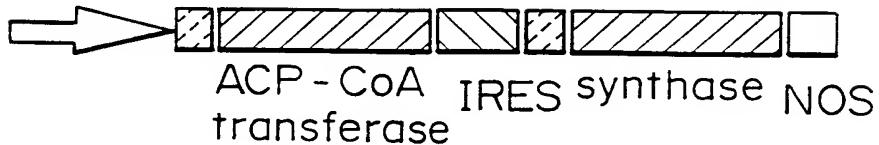
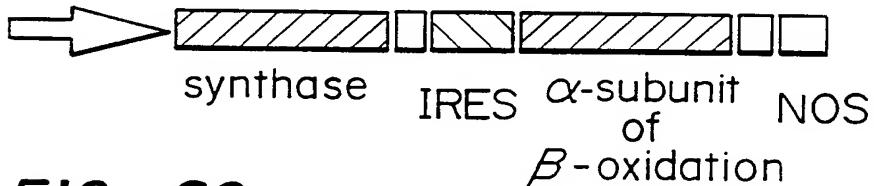
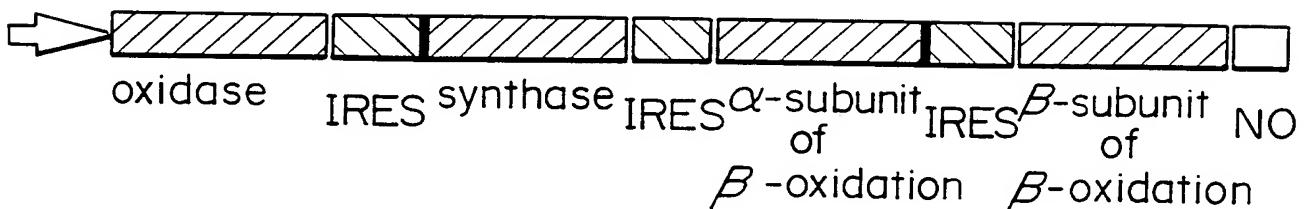


FIG. 4



- promoter
- ▨ peptide signal for chloroplast targeting
- ▨▨▨ coding region of gene to be expressed
- NOS terminator

*FIG. 5*

**FIG. 6A****FIG. 6B****FIG. 6C**

promoter

peptide signal for chloroplast targeting

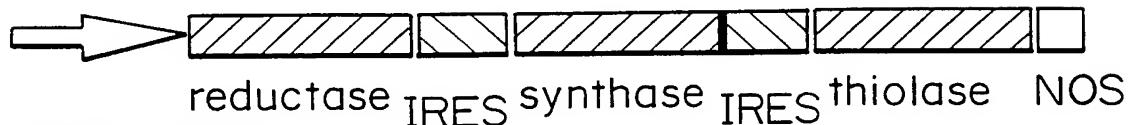
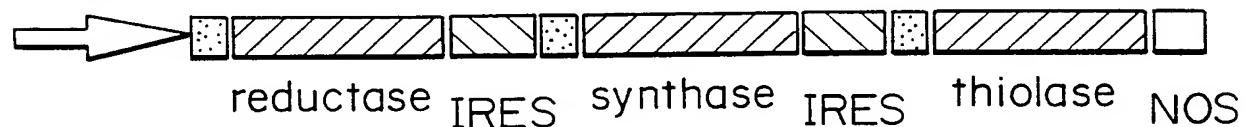
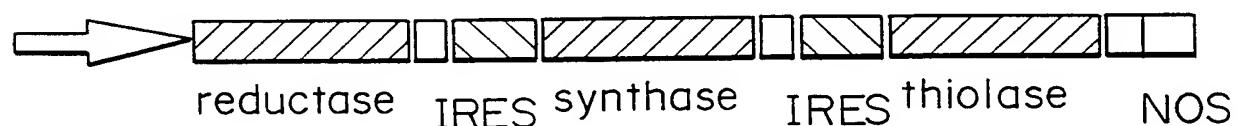
coding region of gene to be expressed

IRES sequence from animal or plant virus

C-terminal peroxisomal targeting signal

NOS terminator

7/9

**FIG. 7A****FIG. 7B****FIG. 7C****FIG. 7D****FIG. 7E****FIG. 7F**

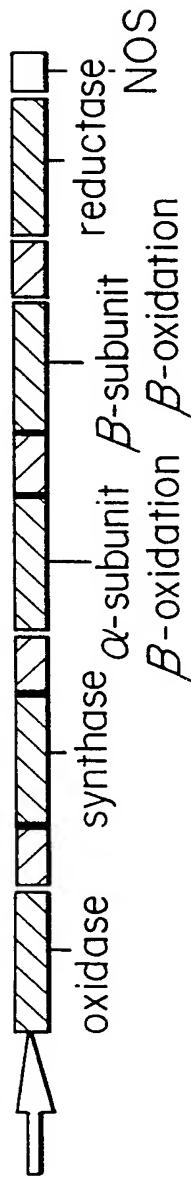
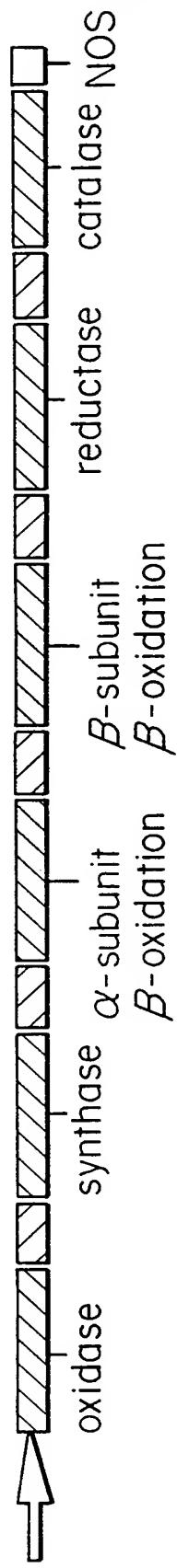
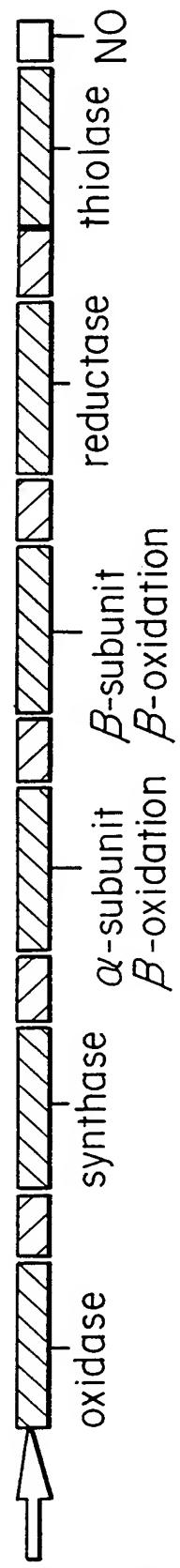
promoter   NOS terminator

peptide signal for chloroplast targeting

coding region of gene to be expressed

IRES sequence from animal or plant virus

C-terminal peroxisomal targeting signal

**FIG. 8A****FIG. 8B****FIG. 8C**

↑  
promoter

▨ coding region of gene to be expressed  
 ▨ IRES sequence from animal or plant virus  
 □ NOS terminator

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promoter

gene 1 encoding enolpyruvylshikimate-3-phosphate

IRES sequence from plant or animal virus

gene 2 encoding *Bacillus thuringiensis* toxin

NOS terminator

*FIG. 9*

## SEQUENCE LISTING

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<120> Plant Multi-gene Expression Constructs

<130> MBX 037 PCT

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accaaca 127

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# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/US 00/17197

A. CLASSIFICATION OF SUBJECT MATTER	IPC 7 C12N15/82	C12N15/67	A01H5/00	A01K67/027
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According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, STRAND, WPI Data, PAJ, BIOSIS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 54342 A (KORPELA TIMO ; ATABEKOV JOSEPH (RU); DOROKHOV YURII (RU); IVANOV PE) 3 December 1998 (1998-12-03) see page 9; claims 5,8 the whole document -----	1-3,6,7, 12-18  -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

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- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search

24 October 2000

Date of mailing of the international search report

07/11/2000

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Holtorf, S

## INTERNATIONAL SEARCH REPORT

International Application No PCT/US 00/17197
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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>MITTENDORF VOLKER ET AL: "Synthesis of medium-chain-length polyhydroxyalkanoates in <i>Arabidopsis thaliana</i> using intermediates of peroxisomal fatty acid beta-oxidation"            PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, US,            vol. 95, no. 23,            10 November 1998 (1998-11-10), pages 13397-13402, XP002143031            ISSN: 0027-8424            the whole document</p> <p>---</p>	
A	<p>WO 95 05472 A (UNIV MICHIGAN)            23 February 1995 (1995-02-23)            the whole document</p> <p>---</p>	
A	<p>WO 98 06854 A (MONSANTO CO)            19 February 1998 (1998-02-19)            abstract, page 4, example 10,</p> <p>---</p>	
A	<p>MADISON LARA L ET AL: "Metabolic engineering of poly(3-hydroxyalkanoates): From DNA to plastic."            MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS,            vol. 63, no. 1, March 1999 (1999-03),            pages 21-53, XP002150883            ISSN: 1092-2172            cited in the application</p> <p>---</p>	
A	<p>SHEEN JEN: "Protein phosphatase activity is required for light-inducible gene expression in maize."            EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL,            vol. 12, no. 9, 1993, pages 3497-3505,            XP002150884            ISSN: 0261-4189            cited in the application</p> <p>---</p>	
P,X	<p>SKULACHEV M V ET AL: "Internal initiation of translation directed by the 5'-untranslated region of the tobamovirus subgenomic RNA I2."            VIROLOGY,            vol. 263, no. 1,            10 October 1999 (1999-10-10), pages 139-154, XP002150885            ISSN: 0042-6822            the whole document</p> <p>---</p> <p>-/-</p>	1-3,6, 12-18

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 00/17197

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CA 2 244 959 A (ABOUHAIDAR, M.; HEFFERON, K.; MEULEWATER, F.; VAN ELDIK, G.) 22 March 2000 (2000-03-22) page 6, 9, 26; examples 3, claims -----	1,2,6,7, 12-18

# INTERNATIONAL SEARCH REPORT

## Information on patent family members

International Application No

PCT/US 00/17197

Patent document cited in search report	Publication date	Patent family member(s)			Publication date
WO 9854342	A 03-12-1998	FI 972293	A	01-12-1998	
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		EP 1017834	A	12-07-2000	
WO 9505472	A 23-02-1995	US 5610041	A	11-03-1997	
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CA 2244959	A	NONE			